JC07 Rec'd PCT/PTO 23 JAN 2002

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TRANSMITTAL LETTER TO THE UNITED STATES ATTORNEY'S DOCKET NUMBER 50521

DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371 U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 10/031702

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/07211	**26 July 2000**	**27 July 1999**

TITLE OF INVENTION: EPOXIDE HYDROLASES FROM STREPTOMYCES

APPLICANT(S) FOR DO/EO/US Frank ZOCHER, Markus ENZELBERGER, Rolf D. SCHMID, Wolfgang WOHLLEBEN, Bernhard HAUER

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. /X/ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.

2. / / This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.

3. /X/ This express request to begin national examination procedures (35 U.S.C.371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. /x/ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. /X/ A copy of the International Application as filed (35 U.S.C. 371(c)(2)).

   a./X/ is transmitted herewith (required only if not transmitted by the International Bureau).
   b./ / has been transmitted by the International Bureau.
   c./ / is not required, as the application was filed in the United States Receiving Office (RO/US0).

6. /X/ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. / / Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).

   a./ / are transmitted herewith (required only if not transmitted by the International Bureau).
   b./ / have been transmitted by the International Bureau.
   c./ / have not been made; however, the time limit for making such amendments has NOT expired.
   d./ / have not been made and will not be made.

8. / / A translation of the amendments to the claims under PCT Article 19(35 U.S.C. 371(c)(3)).

9. /X / An oath or declaration of the inventor(s)(35 U.S.C. 171(c)(4)).

10./ / A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11./ / An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12./ x / An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13./ X / A FIRST preliminary amendment.
/ / A SECOND or SUBSEQUENT preliminary amendment.

14./ / A substitute specification.

15./ / A change of power of attorney and/or address letter.

16./x / Other items or information.
International Search Report
International Preliminary Examination Report

531 Rec'd PCT/PTC 23 JAN 2002

U.S. Appln. No. (If Known) 10/031702 | INTERNATIONAL APPLN. NO. PCT/EP00/07211 | ATTORNEY'S DOCKET NO. 50521

	CALCULATIONS	PTO USE ONLY
17. /X/ The following fees are submitted BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO..$890.00	890.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482)..$710.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))..........................$740.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO ....$ 1,040.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied pro -visions of PCT Article 33(2)-(4).....................$100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT = $	890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than / / 20 / /30 months from the earliest claimed priority date (37 CFR 1.492(e)).		

Claims	Number Filed	Number Extra	Rate		
Total Claims	15	-20	X$18.		
Indep.Claims	4	-3	X$84.	84.00	
Multiple dependent claim(s)(if applicable)			+280.		
TOTAL OF ABOVE CALCULATION			=	**974.00**	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).					
SUBTOTAL			=	974.00	
Processing fee of $130. for furnishing the English translation later than / /20 / /30 months from the earliest claimed priority date (37 CFR 1.492(f)). +					
TOTAL NATIONAL FEE			=	**974.**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) $40.00 per property =				40.	
TOTAL FEES ENCLOSED			=	$1,014.00	
			Amount to be refunded:	$	
			Charged	$	

a./X/ A check in the amount of $1,014. to cover the above fees is enclosed.

b./ / Please charge my Deposit Account No.______ in the amount of $______ to cover the above fees. A duplicate copy of this sheet is enclosed.

c./X/ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **11-0345**. A duplicate copy of this sheetis enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.

Herbert B Keil
SIGNATURE

Herbert B. Keil
NAME
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SEND ALL CORRESPONDENCE TO:
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of
ZOCHER et al.

BOX PCT

International Application
PCT/EP 00/07211

Filed: July 26, 2000

For: EPOXIDE HYDROLASES FROM STREPTOMYCES

PRELIMINARY AMENDMENT

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

Prior to examination, kindly amend the above-identified application as follows:

IN THE CLAIMS

Kindly amend the claims as shown on the attached sheets.

REMARKS

The claims have been amended to eliminate multiple dependency and to place them in better form for U.S. filing. No new matter is included.

A clean copy of the claims is attached.

Favorable action is solicited.

Respectfully submitted,

KEIL & WEINKAUF

HBKeil

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MARKED VERSION OF AMENDED CLAIMS - OZ 50521

5. A process for separating epoxide enantiomer mixtures, which comprises

   a) incubating an epoxide enantiomer mixture, which comprises an epoxide hydrolase substrate, with an epoxide hydrolase as claimed in <u>claim 1</u> [one of claims 1 to 3], a microorganism of the genus Streptomyces, an epoxide hydrolase-containing homogenate thereof, or a fraction of this homogenate;

   b) converting the enantiomeric mixture, preferably until the reaction equilibrium is established; and

   c) fractionating the reaction mixture.

11. A method as claimed in <u>claim 7</u> [one of claims 7 to 10], wherein the epoxide-containing substrate is styrene oxide, 3-phenylglycidate, hexane-1,2-oxide and/or indene oxide in enantiomeric pure form or an enantiomeric mixture.

12. A screening method for detecting microorganisms having epoxide hydrolase activity and/or having the ability for the enantioselective hydrolysis of epoxides, comprising a detection method as claimed in <u>claim 7</u> [one of claims 7 to 11].

13. The use of an epoxide hydrolase as claimed in <u>claim 1</u> [one of claims 1 to 3], a microorganism of the genus Streptomyces, an epoxide-hydrolase-containing homogenate thereof or a fraction of this homogenate for the enantioselective hydrolysis of epoxides.

14. The use of an epoxide hydrolase as claimed in <u>claim 1</u> [one of claims 1 to 3], a microorganism of the genus Streptomyces[.], an epoxide-hydrolase-containing

homogenate thereof or a fraction of this homogenate for the enantioselective preparation of hydroxides from the corresponding epoxides.

15. A process for producing epoxide hydrolases (E.C. 3.3.2.3), wherein

    a) a cell homogenate is produce from [form] a culture of a microorganism of the genus Streptomyces;

    b) the homogenate is fractionated, the resultant fractions being tested for epoxide hydrolase activity, if appropriate using a detection method as claimed in claim 7 [one of claims 7 to 11]; and

    c) fractions having epoxide hydrolase activity are combined and if appropriate further fractionated.

10/031702
531 Rec'd PCT/PTO 23 JAN 2002

CLEAN VERSION OF AMENDED CLAIMS - OZ 50521

5. A process for separating epoxide enantiomer mixtures, which comprises
   a) incubating an epoxide enantiomer mixture, which comprises an epoxide hydrolase substrate, with an epoxide hydrolase as claimed in claim 1, a microorganism of the genus Streptomyces, an epoxide hydrolase-containing homogenate thereof, or a fraction of this homogenate;
   b) converting the enantiomeric mixture, preferably until the reaction equilibrium is established; and
   c) fractionating the reaction mixture.

11. A method as claimed in claim 7, wherein the epoxide-containing substrate is styrene oxide, 3-phenylglycidate, hexane-1,2-oxide and/or indene oxide in enantiomeric pure form or an enantiomeric mixture.

12. A screening method for detecting microorganisms having epoxide hydrolase activity and/or having the ability for the enantioselective hydrolysis of epoxides, comprising a detection method as claimed in claim 7.

13. The use of an epoxide hydrolase as claimed in claim 1, a microorganism of the genus Streptomyces, an epoxide-hydrolase-containing homogenate thereof or a fraction of this homogenate for the enantioselective hydrolysis of epoxides.

14. The use of an epoxide hydrolase as claimed in claim 1, a microorganism of the genus Streptomyces, an epoxide-hydrolase-containing homogenate thereof or a fraction of this homogenate for the enantioselective preparation of hydroxides from the corresponding epoxides.

CLEAN VERSION OF AMENDED CLAIMS - OZ 50521

15. A process for producing epoxide hydrolases (E.C. 3.3.2.3), wherein

   a) a cell homogenate is produce from a culture of a microorganism of the genus Streptomyces;

   b) the homogenate is fractionated, the resultant fractions being tested for epoxide hydrolase activity, if appropriate using a detection method as claimed in claim 7; and

   c) fractions having epoxide hydrolase activity are combined and if appropriate further fractionated.

10/031702
531 Rec'd PCT. 23 JAN 2002

CLAIMS AS FILED - OZ 50521

1. An epoxide hydrolase (E.C. 3.3.2.3) microorganism of the genus Streptomyces.

2. An epoxide hydrolase as claimed in claim 1, having at least one of the following properties:

   a) hydrolytic epoxide cleavage of a styrene oxide and of at least one other compound selected from ethyl 3-phenylglycidates, n-hexane- 1,2-oxides, n-decane-1,2-oxides and indene oxides;

   b) conversion of a racemate of styrene oxide with an enantioselectivity $E \geq 2$ to give (S)-phenyl- 1,2-ethanol.

3. An epoxide hydrolase isolated from bacteria of the genus Streptomyces, in particular from the species S. griseus, S. thermovulgaris, S. antibioticus, S. arenae and S. fradiae, preferably from the strains Streptomyces griseus (DSM 40236 and DSM 13447), Streptomyces thermovulgaris (DSM 40444 and DSM 13448) , Streptomyces antibioticus Tü4 (DSM 12925) Streptomyces arenae Tü495 (DSM 40737 and DSM 12134) or Streptomyces fradiae Tü 27 (DSM 12131) .

4. A Streptomyces antibioticus Tü4 deposited at the DSMZ under the Deposit Number DSM 12925.

5. A process for separating epoxide enantiomer mixtures, which comprises

   a) incubating an epoxide enantiomer mixture, which comprises an epoxide hydrolase substrate, with an epoxide hydrolase as claimed in claim 1, a microorganism of the genus Streptomyces, an epoxide hydrolase-

containing homogenate thereof, or a fraction of this homogenate;

b) converting the enantiomeric mixture, preferably until the reaction equilibrium is established; and

c) fractionating the reaction mixture.

6. A process as claimed in claim 5, wherein an enantiomeric mixture of an epoxide is converted, which mixture is selected from styrene oxides, 3-phenylglycidates, hexane-1,2-oxides, decane- 1,2-oxides and indene oxides.

7. A detection method for epoxide hydrolase, which comprises

a) incubating an analyte in which epoxide hydrolase activity is suspected with an epoxide-containing substrate for the hydrolase under reaction conditions;

b) carrying out a color reaction with unreacted epoxide in the presence of 4-nitrobenzylpyridine (NBP); and

c) analyzing the solution from step b) for decrease in pigment concentration, relative an epoxide hydrolase-free control solution.

8. A method as claimed in claim 7, wherein the relative decrease in pigment concentration is determined quantitatively and the epoxide hydrolase activity in the analyte is determined therefrom.

9. A method as claimed in claim 8, wherein the analyte is a microorganism, a homogenate therefrom or a fraction of this homogenate

10. A process as claimed in claim 9, wherein the microorganism is a bacterium of

the genus Streptomyces.

11. A method as claimed in claim 7, wherein the epoxide-containing substrate is styrene oxide, 3-phenylglycidate, hexane-1,2-oxide and/or indene oxide in enantiomeric pure form or an enantiomeric mixture.

12. A screening method for detecting microorganisms having epoxide hydrolase activity and/or having the ability for the enantioselective hydrolysis of epoxides, comprising a detection method as claimed in claim 7.

13. The use of an epoxide hydrolase as claimed in claim1, a microorganism of the genus Streptomyces, an epoxide-hydrolase-containing homogenate thereof or a fraction of this homogenate for the enantioselective hydrolysis of epoxides.

14. The use of an epoxide hydrolase as claimed in claim 1, a microorganism of the genus Streptomyces, an epoxide-hydrolase-containing homogenate thereof or a fraction of this homogenate for the enantioselective preparation of hydroxides from the corresponding epoxides.

15. A process for producing epoxide hydrolases (E.C. 3.3.2.3), wherein

    a) a cell homogenate is produced from a culture of a microorganism of the genus Streptomyces;

    b) the homogenate is fractionated, the resultant fractions being tested for epoxide hydrolase activity, if appropriate using a detection method as claimed in one of claims 7 to 11; and

    c) fractions having epoxide hydrolase activity are combined and if appropriate further fractionated.

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10/031702

531 Rec'd PCT/PTO 23 JAN 2002

Epoxide hydrolases from Streptomyces

The invention relates to improved epoxide hydrolases which can be isolated from bacteria of the genus Streptomyces, a novel process for the enzymatic separation of epoxide enantiomer mixtures, a novel detection method for epoxide hydrolase activity, a screening method for detecting epoxide hydrolase activity and the use of bacteria of the genus Streptomyces and the resultant epoxide hydrolases for enantioselective epoxide hydrolysis.

The increasing importance of enantiomerically pure compounds, especially in the pharmaceutical and agrochemical industries, requires reliable and economic access to optically active substances. To prepare enantiomerically pure diols and epoxides, a number of methods are available.

In asymmetric chemical synthesis of epoxides, synthesis starts from a prochiral compound. By using a chiral reagent, for example a chiral peracid, a chiral dioxirane or oxaziridine or a chiral borate, a chiral auxiliary or a chiral, metallic or nonmetallic catalyst, a chiral epoxide is formed. The best known pathway for synthesizing chiral epoxides is the Sharpless epoxidation of alkenols, for example allyl alcohols, with hydroperoxides in the presence of transition metal catalysts.

Preparation of enantiomerically pure diols by a biochemical pathway is also known. Microorganisms having epoxide hydrolase activity catalyze the regiospecific and enantiospecific hydrolysis of epoxides. They cleave the ether bond in epoxides, forming diols. Some bacterial strains have already been described which enable a broad selection of racemic epoxides to be hydrolyzed enantioselectively. However, the number of known epoxide hydrolases and their

application to organic synthesis has been restricted to date. Known strains having epoxide hydrolase activity are, for example, Aspergillus niger LCP521, Bacillus sulfurescent ATCC 7159, Rhodococcus species NCIMB 11216 and others.

However, the known strains having epoxide hydrolase activity often have a restricted substrate spectrum and low reaction rates. Also, the enantioselectivities which can be achieved using these strains are frequently too low [Grogan, G., et al., FEMS Microbiology Lett. 141 (1996), 239 - 243; Kroutil, W., et al., Tetrahedron Lett. (1996), 8379 - 8382]. The known strains are difficult to manipulate genetically and some are difficult to culture. Therefore, to date, only two epoxide hydrolases are available in recombinant form in E.coli [Corynebacterium sp. C12, (Misawa, E., et al., Eur. J. Biochem. 253 (1998), 173 - 183) and Agrobacterium radiobacter AD1 (Rink, R., et al., J. Biol. Chem. 272 (1997), 14650 - 14657)]. Even purification of the epoxide hydrolases obtained from the microorganisms has to date only been described for Rhodococcus species NCIMB 11216 [Faber, K., et al., Biotechnology Lett. 17 (1995), 893 - 898] and Norcardia EH 1 [Kroutil, W., et al J. Biotechnol. 61 (1998), 143 - 150]. This was highly complex in both cases. The enrichment of epoxide hydrolases from Corynebacterium sp. C12 is described by Misawa, E., et al., Eur. J. Biochem. 253, (1998) 173-183.

In addition, the search for novel epoxide hydrolase-containing microorganisms is made difficult owing to the fact that screening for novel epoxide-hydrolase-producing microorganisms, for example in collections of microbiological strains, has hitherto been highly time-consuming. This is due to the fact that, for screening, usually methods are used in which the individual batches must be worked up and analyzed individually by gas chromatography or liquid chromatography.

It is a first object of the invention, therefore, to provide novel epoxide hydrolases having an expanded substrate spectrum and/or improved reactivity and/or improved enantioselectivity. In addition, the novel epoxide hydrolases should be more readily accessible, in particular, because they can be isolated from nonpathogenic organisms which can be readily cultured, and, in addition, if appropriate are readily accessible to methods of molecular biology.

It is a second object of the invention to provide a method for the more rapid and simpler detection of epoxide hydrolase, which should also allow improved screening for epoxide-hydrolase-producing micro-organisms.

It is a third object of the invention to provide an improved biochemical process for separating epoxide enantiomer mixtures and thus an improved process for the enantioselective reaction of epoxides which permits a simpler route to the enantiomerically pure diols and/or epoxides.

It is a fourth object of the invention to provide novel epoxide-hydrolase-producing microorganisms.

We have found that the above first object is achieved, surprisingly, by providing epoxide hydrolases (E.C. 3.3.2.3) from microorganisms of the genus Streptomyces. Epoxide hydrolase activity has not previously been described in microorganisms of this genus.

The inventive epoxide hydrolases have at least one of the following advantageous properties, compared with previously known epoxide hydrolases:

- improved enantioselectivity in the resolution of enantiomeric epoxides;
- improved (expanded) substrate spectrum;

- improved reactivity;
- enhanced accessibility to methods of molecular biology;
- improved biochemical accessibility because the microorganisms are easier to culture.

For the purposes of the invention, "improved enantio-selectivity" is when, for substantially the same conversion rate, a higher enantiomeric excess is achievable.

For the purposes of the invention, an "expanded substrate spectrum" is that racemic mixtures of a plurality of epoxides are converted.

For the purposes of the invention, an "improved reactivity" is that the reaction takes place with a higher space-time yield.

The invention relates in particular to those epoxide hydrolases from Streptomyces that have at least one of the following properties:

a) hydrolytic epoxide cleavage of a styrene oxide, for example styrene oxide or a derivative thereof which is monosubstituted or polysubstituted on the phenyl ring or epoxide ring, such as in particular a styrene oxide which is monosubstituted in the meta or para position by nitro or halogen, in particular chlorine or bromine, and at least one further compound selected from the group consisting of ethyl 3-phenylglycidate, n-hexane-1,2-oxide, n-decane-1,2-oxide and indene oxide, which can be unsubstituted or monosubstituted or polysubstituted by substituents preferably in accordance with the above definition;

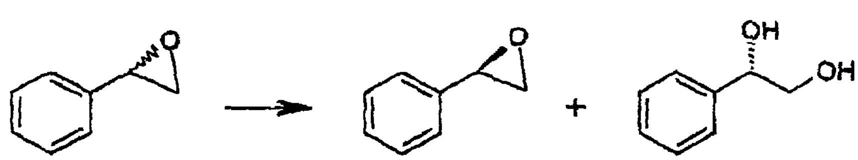
b) conversion of a racemate of styrene oxide with an enantioselectivity E ≥ 2, for example ≥ 10, for

instance from 10 to 100, to give (S)-phenyl-1,2-ethanol according to the reaction equation (A) given below, this conversion being able to be carried out using whole cells or a cell homogenate or an enriched or purified enzyme preparation, and preferably taking place in the presence of a cosolvent, for example from 5 to 10% (v/v) DMSO.

According to a preferred embodiment, the epoxide hydrolases provided are those which can be isolated from bacteria of the genus Streptomyces, in particular from the species S. griseus, S. thermovulgaris, S. antibioticus, S. arenae and S. fradiae, preferably from the strains Streptomyces griseus (DSM 40236 and DSM 13447), Streptomyces thermovulgaris (DSM 4044 and DSM 13448), Streptomyces arenae Tü (DSM 40737 and DSM 12134) Streptomyces antibioticus Tü4 (DSM 12925) or Streptomyces fradiae Tü 27 (DSM 12131).

Particular preference is given to the epoxide hydrolase which can be isolated from Streptomyces antibioticus Tü4 (DSM 12925). This enzyme is characterized by its pronounced enantioselectivity and the conversion of (R/S)-styrene oxide (I) according to the following reaction equation (A)

Reaction equation (A):



(I) (II) (III)

to (S)-phenyl-1,2-ethanediol (III), with the non-hydrolysis of (R)-styrene oxide (II). Thus this enzyme in a reaction medium containing 10% (v/v) DMSO as solubilizer, catalyzes the above conversion at an enantioselectivity of E = 13 and an enantiomeric excess

ee[%] = 99 for (II) and ee[%] = 14 for (III).

Isolation of the enzyme is described in more detail in the examples. Unless stated otherwise, the enzyme is enriched using standard biochemical methods, for example as described by T.G. Cooper in Biochemische Arbeitsmethoden [Biochemical methods], Verlag Walter de Gruyter, Berlin, New York, (1981). Suitable methods, for example, are purification methods such as precipitation, for example using ammonium sulfate, ion-exchange chromatography, gel chromatography, affinity chromatography, for example immunoaffinity chromatography, and isoelectric focusing, and combinations of these methods.

The invention also relates to a novel Streptomyces strain having the designation Streptomyces antibioticus Tü4, deposited at the DSMZ under the Deposit Number DSM 12925 and variants and mutants of this strain.

The invention also relates to functional analogs of the inventively prepared enzymes, such as variants, alleles and mutants, that have epoxide hydrolase activity and, preferably, have at least one of the abovementioned advantageous properties.

We have found that the above second object was achieved, surprisingly, by providing an optical method of detection for epoxide hydrolase, which comprises

a) incubating an analyte, for example a microorganism culture, in which epoxide hydrolase activity is suspected with an epoxide-containing substrate for the epoxide hydrolase under reaction conditions;

b) chemically reacting unreacted epoxide with 4-nitrobenzylpyridine (NBP), forming a pigment absorbing at 560 nm; and

c) analyzing the solution from step b) for decrease in pigment concentration, relative to an epoxide-hydrolase-free control solution.

The inventive detection method can be carried out qualitatively, for example as a spot test, or quantitatively. In the quantitative method, the relative decrease in pigment concentration is first determined quantitatively, for example photometrically by determining absorption at 560 nm, and the epoxide hydrolase activity in the analyte is determined therefrom.

Suitable analytes are in principle microorganisms per se, for example samples from a freshly taken culture of a bacterium, cell homogenates thereof or fractions of these cell homogenates after purification. Preferably, the test is carried out using whole cells or after digestion of the cells, for example using ultrasound or lysozyme.

For example, the method is applicable to detecting epoxide hydrolase in bacteria of the genus Streptomyces.

Preferably, the detection method is carried out in such a manner that a sample is withdrawn from a freshly taken culture of the microorganism, this is disrupted, freed from cell fragments and an epoxide comprising a substrate for the enzyme to be tested is added and mixed. If required, the reaction conditions can be optimized in the solution by customary measures, for example by adding buffer, adjusting the reaction temperature and the like. Preferably, to improve the solubility of the epoxide in the aqueous reaction medium, a cosolvent is used. Suitable cosolvents are, for example, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), ethanol or acetone. Optimum reaction conditions for epoxide hydrolases from Streptomyces comprise:

Reaction medium:	Sodium phosphate buffer pH 8.0, 0.1 M, 10% (v/v) DMSO
pH range:	6-8
Temperature:	30-37°C
Reaction time:	2-20 hours
Substrate concentration:	from 0.1 to 0.8 molar

The substrate preferably used is styrene oxide, 3-phenylglycidate, hexane-1,2-oxide, decane-1,2-oxide and/or indene oxide in enantiomerically pure form or as enantiomeric mixture.

To develop the color reaction, the pH is then adjusted by adding a base, for example triethylamine. NBP is then added, for example at a concentration in the range from about 3 to 10%, preferably about 5% (w/v) in methoxyethanol. As solubilizer for the pigment formed, for example, triethylene glycol dimethyl ether is added in sufficient quantity. The solution is finally incubated at from about 35 to 45°C, preferably 39°C, and the absorption is then determined at 560 nm, preferably against a reference at 650 nm. From comparison with an enzyme-free control solution, the decrease in absorption and thus the decrease in epoxide can be determined quantitatively.

The invention also relates to screening methods for detecting microorganisms having epoxide hydrolase activity and/or having the ability to hydrolyze epoxides enantioselectively, comprising the above described detection method. This is particularly suitable for systematic study of strain collections or mutant banks, generated by "directed evolution", for epoxide hydrolase activity.

We have found that the above third object was achieved, surprisingly, by providing a process for separating epoxide enantiomer mixtures which comprises

a) incubating an epoxide enantiomer mixture, which comprises an epoxide hydrolase substrate, with an inventive epoxide hydrolase, a microorganism of the genus Streptomyces, an epoxide-hydrolase-containing homogenate thereof or a fraction of this homogenate under reaction conditions;

b) reacting the enantiomer mixture, preferably to achieve a 50% conversion rate; and

c) separating the enantiomer remaining in the reaction mixture from the conversion product and purifying the essentially enantiomerically pure reaction product and/or the essentially enantiomerically pure starting material remaining.

Preferably, enantiomer mixtures of one of the following epoxides is converted: styrene oxide, 3-phenylglycidate, hexane-1,2-oxide, decane-1,2-oxide and indene oxide or substituted analogs of these oxides in accordance with the above definition.

The invention further relates to a process for producing epoxide hydrolases (E.C. 3.3.2.3), which comprises

a) producing a cell homogenate from a culture of a microorganism of the genus Streptomyces;

b) fractionating the homogenate, the resultant fractions being tested for epoxide hydrolase activity, preferably using a detection method based on the color reaction of unreacted epoxide with NBP according to the above definition; and

c) combining fractions having epoxide hydrolase activity and if appropriate further fractionating.

The invention finally relates to the use of an

inventive epoxide hydrolase, a microorganism of the genus Streptomyces, an epoxidehydrolase-containing homogenate thereof or a fraction of this homogenate for the enantioselective hydrolysis of epoxides.

The invention is described in more detail by the examples below and with reference to the accompanying figures. In the drawings:

Figure 1 shows results of measurements of pigment formation from NBP and epoxide at various styrene oxide concentrations. The change in absorption in solutions in the presence of E.coli DH5a by the NBP assay (black circles), without E.coli DH5a cell and without NBP (black squares) and with cell lysate of E.coli DH5a by the NPB assay (black triangles) are plotted, in each case after 45 minutes at the specified styrene oxide concentrations.

Figure 2 shows the time course of racemate resolution of styrene oxide by the epoxide hydrolase from S. antibioticus Tü4 (DSM 12925). ee[%] (R)-styrene oxide (black squares); ee[%] (S)-phenyl-1,2-ethanediol (black circles); conversion rate [%] (black triangles);

Figure 3 shows the effect of various cosolvents on the hydrolysis of styrene oxide by epoxide hydrolase from S. antibioticus Tü4 (DSM 12925); ee[%] (R)-styrene oxide is plotted as a function of cosolvent concentration (%(v/v)); acetone (black squares); DMSO (black circles); DMF (black triangles).

Example 1: Validation of a test system for epoxide hydrolase

a) Styrene oxide as substrate

An epoxide hydrolase-free organism (E.coli DH5a) was cultured in 2 ml cultures and disrupted. 100 µl aliquots of the resultant cell lysates were distributed over 4 recesses of a microtitre plate. To the cell lysate was added 50 µl of acetone containing various amounts of styrene oxide (1.3 mol/l (stock solution), 0.65 mol/l, 0.32 mol/l, 0.16 mol/l, 0.08 mol/l). The solution was incubated at 30°C for 2 hours. After adding 25 µl of triethylamine (TE), 50 µl of p-nitrobenzylpyridine (NBP, 5% by volume) and 50 µl of triethylene glycol dimethyl ether, it was again incubated at 39°C for 45 minutes and then the absorption was measured at 560 nm against a reference of 650 nm.

The results are shown in Figure 1. The absorption increased linearly with the amount of styrene oxide used. Therefore, the decrease in absorption indicates the presence of an epoxide hydrolase activity.

b) 3-Phenylglycidate as substrate

Example 1a) was repeated, except that the substrate used was 3-phenylglycidate. In this case also, there was a linear relationship between absorption and the remaining amount of unhydrolyzed epoxide.

c) Hexane-1,2-oxide as substrate

Example 1a) was repeated, except that the substrate used was hexane-1,2-oxide. In this case also there was a linear relationship between absorption and the remaining amount of unhydrolyzed epoxide.

d) Indene oxide as substrate

Example 1a) was repeated, except that the substrate

used was indene oxide. In this case also there was a linear relationship between absorption and the remaining amount of unhydrolyzed epoxide.

Example 2: Screening various Streptomyces strains for epoxide hydrolase activity using styrene oxide as substrate

In a similar manner to Example 1, various deposited Streptomyces strains were tested, and also, as negative control, a bacterial strain of the genus Rhodococcus sp. (NCIMB 11216). The microtitre plate wells which contained the negative control exhibited an intensive color reaction (blue coloration), whereas in wells containing Streptomyces antibioticus Tü4 (DSM 12925) and Streptomyces fradiae Tü27 (DSM 12131) the highest epoxide hydrolase activity was found (results not shown).

Example 3: Culturing the strain Streptomyces antibioticus Tü4

The organism was cultured both in 250 ml shake flasks and in a 30 l fermenter; the pH was not monitored.

a) 250 ml fermentation: the culture time at 30°C on the 250 ml scale was from 48 to 72 hours.

b) 30 l fermentation: 2 shake flasks each containing 1 l of malt medium [10 g of malt extract, 4 g of yeast, made up to 1 l with tap water and autoclaved, sterile-filtered glucose (4 g/l final concentration) was added after autoclaving] were inoculated with spores of Streptomyces antibioticus Tü4; the cultures were then shaken for 48 hours (30°C, 210 rpm). 2 l of this preliminary culture were used to inoculate 20 l of malt medium (addition of 0.5 l of 20% strength by weight glucose solution to 20 l of malt

extract/yeast solution), prior homogenization (glass homogenizer, Braun Chemie) being recommended for colony growth as disperse as possible. The culture was stirred for 24 hours at 30°C in the 30 l fermenter (17 l/min of O_2, 200 rpm), then the fermentation was terminated. The cells were centrifuged off at 4000 rpm for 30 minutes and washed with TE buffer (pH 7.3, 1 mmol/l of EDTA. The moist biomass was 318 g. The cells were resuspended in 400 ml of TE buffer (pH 7.3, 1 mmol/l of EDTA).

Example 4: Enrichment of epoxide hydrolase from S. antibioticus Tü4

a) Cell disruption:

For the experiments with disrupted cells, the cultured cells were removed by filtration or centrifugation and washed twice with phosphate buffer (0.1 mol/l of NaCl, 10 mmol/l of EDTA). A suspension (10% w/v) was then prepared in the same buffer additionally containing 10 mg/ml of lysozyme. The suspension was incubated for 1 hour at 30°C and then disrupted twice for 5 minutes each time, with 1 minute in between, with ultrasound (Branson Sonifier W250, output 80W) in an ice bath. The resultant solution was centrifuged off for 60 minutes at 32 500 g and filtered (0.22 mm Sterivex-GP filter, Millipore)

b) Cell extract workup

A column packed with an anion exchanger (Super Q 650M, Toso Haas, 60 mol volume) was equilibrated with 25 ml of tris/HCl buffer (pH 8.1), and then 15 ml of the crude extract were applied. The column was eluted with 2 mol/l NaCl solution containing the following gradients:

1st gradient: 30 ml NaCl solution (0 - 12% by weight),
2nd gradient: 60 ml NaCl solution (12 - 35% by weight),

Wash with 25 ml NaCl solution (100% by weight), reequilibration with 60 ml of tris/HCl buffer.

Flow rate: 4 ml/min, fraction size 4 ml. Protein was determined at 280 nm.

Activity of the individual fractions was determined using the NBP assay. For this, 150 µl from the fractions were added to the wells of a 96-well microtitre plate and the NBP assay was performed as described above using the Biomek 2000 pipetting robot. 50 µl of acetone solution containing styrene oxide (2.6 mol/l) were used in order to reduce the amount of acetone, since the addition of acetone in an amount which is relatively too large can lead to a decrease in enzyme activity. The rest of the experimental procedure was performed in a similar manner to the procedure described above. In the enrichment, all of the epoxide hydrolase activity was detected in one fraction.

Example 5: Conversion of styrene oxide using epoxide hydrolase from S. antibioticus Tü4

a) Reaction procedure

500 µg of styrene oxide were added to 250 ml of the cell-free extract from Example 4 containing 12.5 ml of DMSO as solubilizer. The mixture was incubated at 30°C with uniform shaking (250 rpm).

After 24 hours, the reaction was terminated by adding 30 ml of ethyl acetate, and the aqueous phase was extracted. The organic phase was concentrated under reduced pressure. Gas-chromatographic analysis found an enantiomeric excess of the substrate of (ee_s[%]) of 100 and of the product (ee_p[%]) of 14.

The enantiomeric purity of a chiral substance, which occurs in (R)- and (S) forms, is expressed by the parameter ee (enantiomeric excess). This is defined by the following equation:

$$ee[\%] = [(X_A-X_B)/(X_A+X_B)]^* \, 100$$

where X_A and X_B are the molar fraction of enantiomer A and B, respectively.

The enantiomeric excesses were analyzed by chiral gas chromatography under the following conditions: 75°C isothermal, 130 kPa, on an FS-Cyclodex β-I/P CS-fused silica capillary column (CS-Chromatographie Service GmbH, Langerwehe) (H_2 carrier gas, split 1:100, 0.25 mm × 50 mm).

The product phenyl-1,-2-ethanediol was analyzed under the following conditions: 140°C isothermal under otherwise identical conditions.

The enantioselectivity E of an enzymatic reaction is a constant for an enzyme which is independent of substrate concentration and conversion rate and, for an irreversible reaction without product inhibition, may be calculated using the following formula:

$$\begin{aligned} E &= (V_{max}/K_m)_{(R)\text{-Enantiomer}}/(V_{max}/K_m)_{(S)\text{-Enantiomer}} = \\ &= \{\ln(1-U)(1-ee_s)]\}/\{\ln[(1-U)(1+ee_s)]\} \\ &= \{\ln[(1-U)(1+ee_p)]\}/\{\ln[(1-U)(1-ee_p)]\} \end{aligned}$$

where ee_s is the enantiomeric excess of the substrate and ee_p is the enantiomeric excess of the product and the conversion rate U may be calculated from the formula $U = ee_s/(ee_s+ee_p)$.

Evaluation was performed as described by Chen, C.S. et al., (1982) J.Am.Chem.Soc. 104, 7294.

b) Determination of enantioselectivity

To follow the course of the reaction, 1.5 ml lots of the cell-free extract were shaken with 75 ml of DMSO and 7 ml of styrene oxide in sealable 2 ml reaction vessels at 30°C and extracted with 300 ml of ethyl acetate at predetermined times.
Table 1 shows the results.

Table 1:

Time [h]	ee_s[%]	ee_p[%]	Conversion rate [%]
0.5	-	-	-
1	11	66	15
3.5	23	26	47
5	33	22	59
7	91	21	82
24	100	14	87

The time course of the reaction is shown in the accompanying Figure 2.

Example 6: Hydrolysis of styrene oxide by Streptomyces strains S. fradiae Tü 27 and S. arenae Tü495

Whole cells of a 250 ml culture of the organism were resuspended in 200 ml of sodium phosphate buffer (0.1 M, pH 8). To this were added 500 µl of styrene oxide and 10% (v/v) of DMSO. The reaction solution was shaken at 30°C at 210 rpm in a closed conical flask. Samples (1.5 ml) were taken at various time intervals to monitor the course of the reaction, centrifuged at 14000 rpm for 3 minutes and extracted with 300 µl of diethyl ether. The organic phases were dried using anhydrous sodium sulfate and analyzed by gas chromatography to determine the enantiomeric excess, the conversion rate and the enantioselectivity as described above. The results are summarized in Table 2 below:

Table 2:

Strain	Enantiomeric excess		Conversion rate	Time
	ee_s[a] [%]	ee_p[b] [%]	[%]	[h]
Tü27	70	23	75	48
Tü95	52	38	60	24

a (R)-Styrene oxide

b (S)-Phenyl-1,2-ethanediol

Example 7: Effect of cosolvents on styrene oxide hydrolysis.

Example 6 was repeated, but S. antibioticus Tü4 was used as the microorganism, and DMSO, DMF or acetone were added to the reaction solutions in various amounts. The results are shown in Figure 3. 10% DMSO, 5% acetone and 1-3% DMF led to an increase in enantiomeric excess, and thus in enantioselectivity.

Example 7: Hydrolysis of ethyl 3-phenylglycidate (3-PEG) and n-decane-1,2-oxide by epoxide hydrolase from S. antibioticus Tü4

Example 5 was repeated, but instead of styrene oxide, 3-PEG or n-decane-1,2-oxide was used as substrate.

a) Conversion of 3-PEG:
In contrast to Example 5, 3-PEG was analyzed gas-chromatographically at 60kPa, 130°C (60 min), 180°C (5 min), 10°C/min

b) Conversion of decane-1,2-oxide
The decane-1,2-diol formed was derivatized with acetone and p-toluenesulfonic acid as catalyst in ethyl acetate. Isopropylidenedecane-1,2-diol was analyzed at 120°C under the conditions specified in Example 5.

We claim:

1. An epoxide hydrolase (E.C. 3.3.2.3) from a microorganism of the genus Streptomyces.

2. An epoxide hydrolase as claimed in claim 1, having at least one of the following properties:

   a) hydrolytic epoxide cleavage of a styrene oxide and of at least one other compound selected from ethyl 3-phenylglycidates, n-hexane-1,2-oxides, n-decane-1,2-oxides and indene oxides;

   b) conversion of a racemate of styrene oxide with an enantioselectivity $E \geq 2$ to give (S)-phenyl-1,2-ethanol.

3. An epoxide hydrolase isolated from bacteria of the genus Streptomyces, in particular from the species S. griseus, S. thermovulgaris, S. antibioticus, S. arenae and S. fradiae, preferably from the strains Streptomyces griseus (DSM 40236 and DSM 13447), Streptomyces thermovulgaris (DSM 40444 and DSM 13448), Streptomyces antibioticus Tü4 (DSM 12925) Streptomyces arenae Tü495 (DSM 40737 and DSM 12134) or Streptomyces fradiae Tü 27 (DSM 12131).

4. A Streptomyces antibioticus Tü4 deposited at the DSMZ under the Deposit Number DSM 12925.

5. A process for separating epoxide enantiomer mixtures, which comprises

   a) incubating an epoxide enantiomer mixture, which comprises an epoxide hydrolase substrate, with an epoxide hydrolase as claimed in one of claims 1 to 3, a microorganism of the genus Streptomyces, an epoxide hydrolase-containing

homogenate thereof, or a fraction of this homogenate;

b) converting the enantiomeric mixture, preferably until the reaction equilibrium is established; and

c) fractionating the reaction mixture.

6. A process as claimed in claim 5, wherein an enantiomeric mixture of an epoxide is converted, which mixture is selected from styrene oxides, 3-phenylglycidates, hexane-1,2-oxides, decane-1,2-oxides and indene oxides.

7. A detection method for epoxide hydrolase, which comprises

a) incubating an analyte in which epoxide hydrolase activity is suspected with an epoxide-containing substrate for the epoxide hydrolase under reaction conditions;

b) carrying out a color reaction with unreacted epoxide in the presence of 4-nitro-benzylpyridine (NBP); and

c) analyzing the solution from step b) for decrease in pigment concentration, relative to an epoxide hydrolase-free control solution.

8. A method as claimed in claim 7, wherein the relative decrease in pigment concentration is determined quantitatively and the epoxide hydrolase activity in the analyte is determined therefrom.

9. A method as claimed in claim 8, wherein the analyte is a microorganism, a homogenate therefrom

or a fraction of this homogenate.

10. A process as claimed in claim 9, wherein the microorganism is a bacterium of the genus Streptomyces.

11. A method as claimed in one of claims 7 to 10, wherein the epoxide-containing substrate is styrene oxide, 3-phenylglycidate, hexane-1,2-oxide and/or indene oxide in enantiomeric pure form or an enantiomeric mixture.

12. A screening method for detecting microorganisms having epoxide hydrolase activity and/or having the ability for the enantioselective hydrolysis of epoxides, comprising a detection method as claimed in one of claims 7 to 11.

13. The use of an epoxide hydrolase as claimed in one of claims 1 to 3, a microorganism of the genus Streptomyces, an epoxide-hydrolase-containing homogenate thereof or a fraction of this homogenate for the enantioselective hydrolysis of epoxides.

14. The use of an epoxide hydrolase as claimed in one of claims 1 to 3, a microorganism of the genus Streptomyces., an epoxide-hydrolase-containing homogenate thereof or a fraction of this homogenate for the enantioselective preparation of hydroxides from the corresponding epoxides.

15. A process for producing epoxide hydrolases (E.C. 3.3.2.3), wherein

a) a cell homogenate is produced form a culture of a microorganism of the genus Streptomyces;

b) the homogenate is fractionated, the resultant

fractions being tested for epoxide hydrolase activity, if appropriate using a detection method as claimed in one of claims 7 to 11; and

c) fractions having epoxide hydrolase activity are combined and if appropriate further fractionated.

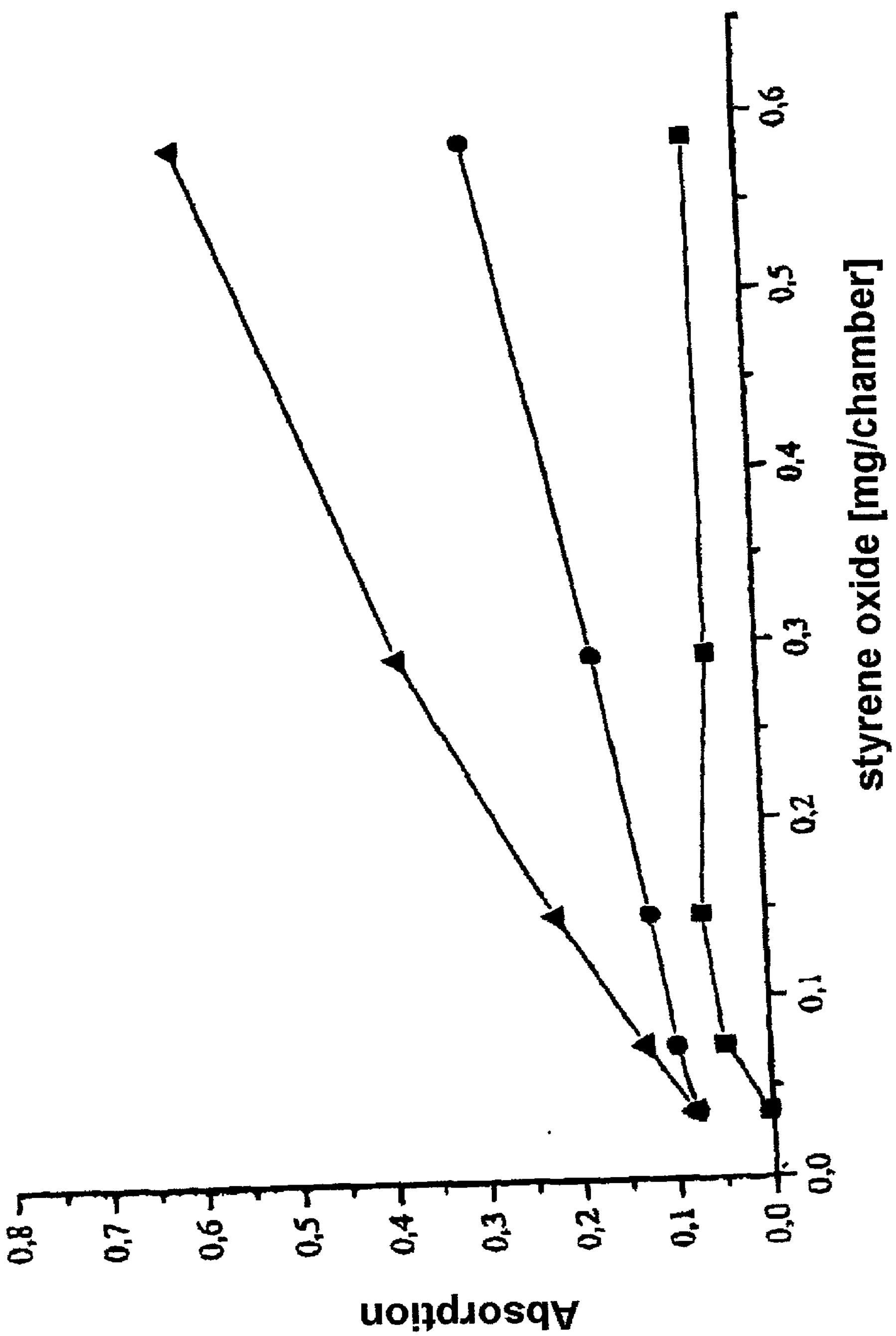
Abstract

Epoxide hydrolases from Streptomyces

A description is given of epoxide hydrolases from bacteria of the genus Streptomyces, a novel process for the enzymatic separation of epoxide enantiomeric mixtures, of a novel detection method for epoxide hydrolase activity, a screening method for detecting epoxide hydrolase activity and the use of bacteria of the genus Streptomyces and of the resultant epoxide hydrolases for enantioselective epoxide hydrolysis.

10/031702

Fig.1



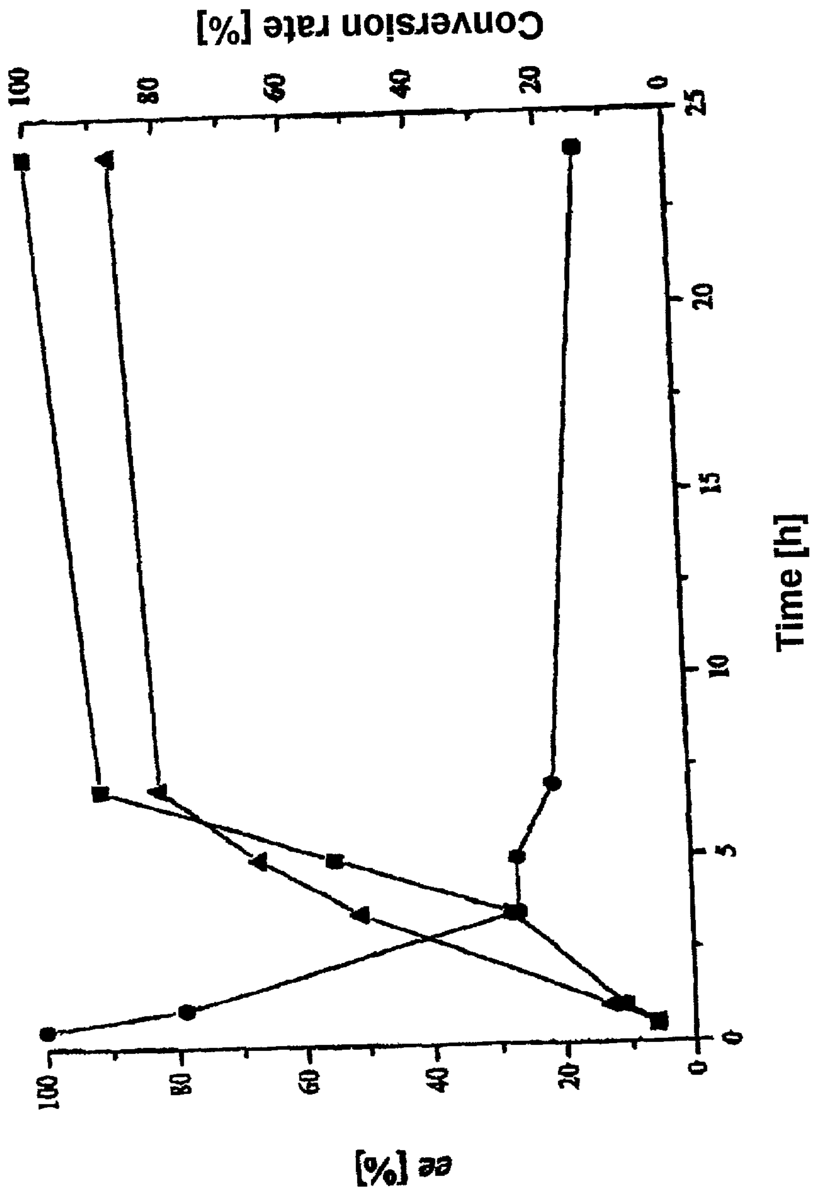

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Fig. 2

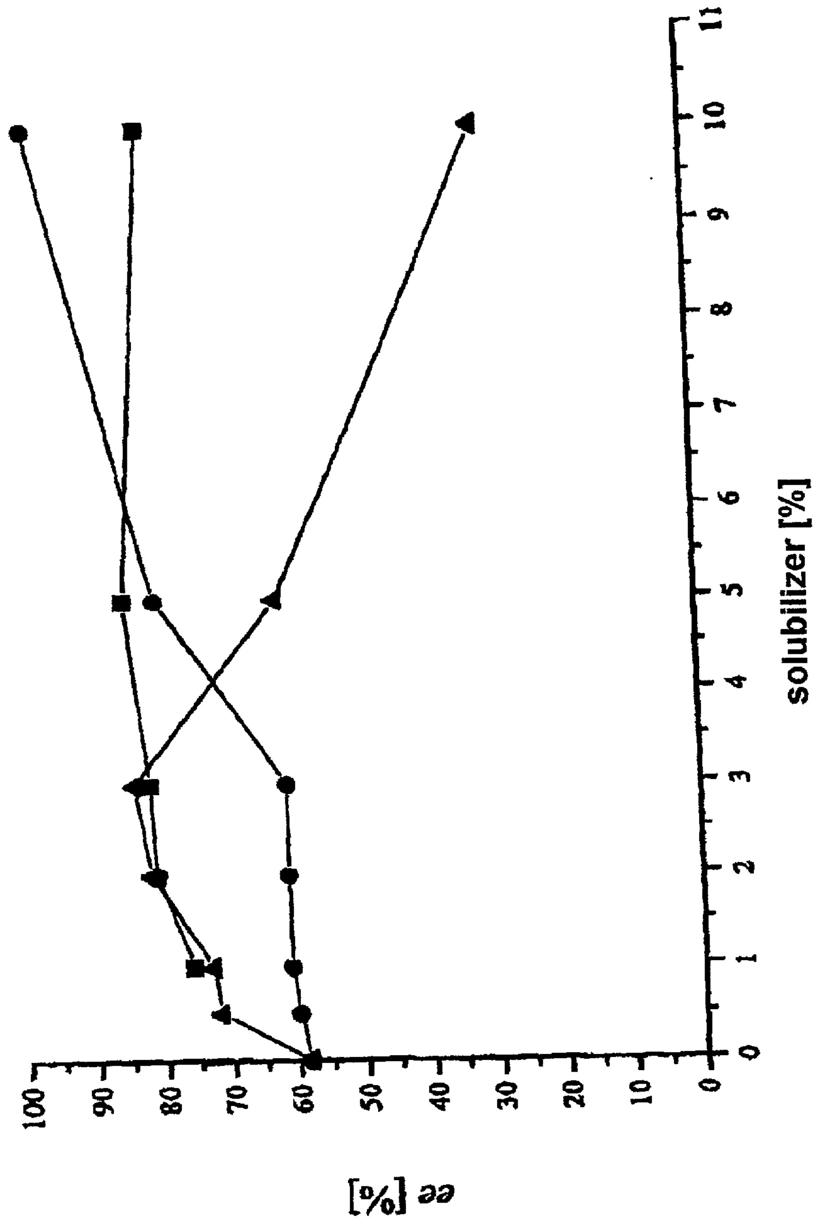

Fig. 3

Declaration, Power of Attorney

We (I), the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name.

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Epoxide hydrolases from Streptomyces

the specification of which

[x] is attached hereto.

[ ] was filed on ______________________________ as

Application Serial No. ______________________________

and amended on ______________________________.

[x] was filed as PCT international application

Number PCT/EP00/07211

on July 26, 2000

and was amended under PCT Article 19

on ______________________________ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above–identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)–(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	**Country**	**Day/Month/Year**	**Priority Claimed**	
19935113.9	*Germany*	*27 July 1999*	[x] Yes	[ ] No

pduskpct001 - 25

We (I) hereby claim the benefit under Title 35, United States Codes, § 119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	**Filing Date**	**Status (pending, patented, abandoned)**

And we (I) hereby appoint **Messrs. HERBERT. B. KEIL**, Registration Number 18,967; and **RUSSEL E. WEINKAUF**, Registration Number 18,495; the address of both being Messrs. Keil & Weinkauf, 1101 Connecticut Ave., N.W., Washington, D.C. 20036 (telephone 202–659–0100), our attorneys, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to sign the drawings, to receive the patent, and to transact all business in the Patent Office connected therewith.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

pdtuskpct001 - 25

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